

1995), and the LPS captured by LPS-binding protein (LBP) is transferred to CD 14 on the surface of a cell (Science 249, 1431-1433, 1990, Annu. Rev. Immunol. 13, 437-457, 1995). The present inventors generated TLR4 knockout mice and reported that the TLR4 knockout mice lack the ability to respond to LPS, a main component of the outer membrane of the Gram-negative bacteria (J. Immunol. 162, 3749-3752, 1999), and also generated TLR2 knockout mice and reported that macrophages derived from TLR2 knockout mice showed low levels of response to cell wall of Gram-negative bacteria or peptidoglycan, a component of the Gram-negative bacteria (Immunity 11, 443-451, 1999).

On the other hand, from the fact that the oligonucleotides comprising bacterial DNA (DNA derived from bacteria) or an unmethylated CpG sequence stimulate immune cells of mice or human (Trends Microbiol. 4, 73-76, 1996, Trends Microbiol. 6, 496-500, 1998), and stimulate a T helper 1 cells (Th1)-like inflammatory response dominated by the release of IL-12 and IFN γ (EMBO J. 18, 6973-6982, 1999, J. Immunol. 161, 3042-3049, 1998, Proc. Natl. Acad. Sci. USA 96, 9305-9310, 1999), it is advocated that the oligonucleotides comprising CpG sequence are possibly used as an adjuvant in vaccine strategies including vaccines to cancer, allergy and infectious diseases (Adv. Immunol. 73, 329-368, 1999, Curr. Opin. Immunol. 12, 35-43, 2000, Immunity 11, 123-129, 1999). Although its effects have been expected in the clinical practice in this way, the molecular mechanism by which bacterial DNA comprising an unmethylated CpG sequence activates immune cells is unclear.

Although the DNA derived from bacteria comprising an unmethylated CpG motif activates immune cells significantly and induces response by Th1 as mentioned above, the activities at the molecular level are not well understood. The goal of the present invention is to provide a receptor protein TLR9, a member of TLR family specifically recognizing bacterial DNA comprising an unmethylated CpG sequence, the DNA encoding it, and the artificial animal models useful in examining response of host immune cells to bacterial infectious diseases, which elucidate effects of oligonucleotides comprising an unmethylated CpG sequence of bacterial DNA at the molecular level.

As a member of the mammalian TLR family, a pattern recognition receptor recognizing common structures of bacteria, relevant to innate immune recognition, six members (TLR1 to 6) have been publicized until now (Nature 388, 384-397, 1997, Proc. Natl. Acad. Sci. USA, 95, 588-593, 1998, Gene 231, 59-65, 1999), and TLR7 and TLR8, two novel members, are registered in GenBank (Registration No: AF240467 and AF246971). Although full-length

cDNA is also found out for TLR9, and is registered in GenBank (Registration No: AF245704), its function has not been known.

The present inventors screened the DNA encoding TLR family member receptor proteins specifically recognizing bacterial DNA comprising an unmethylated CpG sequence on BLAST search, screened a number of sequence tagged (EST) clones highly homologous to various TLR already identified, isolated full-length cDNA from mouse macrophage cDNA library by using the fragments as a probe. We also isolated the human cDNA in the same manner. Next, the sequences of bases of the cDNA were examined, and it was confirmed that it is TLR9, in which regions conserved in the TLR family such as LRR and TIR domains are present. We generated TLR9 knockout mice, showed that TLR9 is a receptor protein to the oligonucleotides comprising an unmethylated CpG sequence of bacterial DNA and completed the invention.

DISCLOSURE OF THE INVENTION

The present invention relates to DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence (claim 1), the protein according to claim 1 wherein a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is either of the following proteins (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No: 2, or (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No: 2, and having reactivity against bacterial DNA having an unmethylated CpG sequence (claim 2), the DNA according to claim 1 comprising the sequence of bases shown in Seq. ID No: 1 or its complementary sequence, or part or whole of the sequences (claim 3), the DNA according to claim 1 which hybridizes with the DNA comprising a gene according to claim 3 under a stringent condition (claim 4), the protein according to claim 1 wherein a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is either of the following proteins (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No: 4, or (b) a protein comprising a sequence of amino acids wherein one or more of amino acid are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No: 4, and having reactivity against bacterial DNA having an unmethylated CpG sequence (claim 5), the DNA according to claim 1 comprising the sequence of bases shown in Seq. ID

No: 3 or its complementary sequence, or part or whole of the sequences (claim 6), and the DNA according to claim 1 which hybridizes with the DNA comprising the gene according to claim 6 under a stringent condition (claim 7).

The present invention also relates to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence (claim 8), the protein according to claim 8 comprising the sequence of amino acids shown in Seq. ID No: 2 (claim 9), the protein according to claim 8 comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in the sequence of amino acids shown in Seq. ID No: 2 (claim 10), the protein according to claim 8 comprising the sequence of amino acids shown in Seq. ID No: 4 (claim 11), and the protein according to claim 8 comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in the sequence of amino acids shown in Seq. ID No: 4 (claim 12).

The present invention also relates to a fusion protein comprising the protein according to any one of claims 8 to 12 fused with a marker protein and/or a peptide tag (claim 13), an antibody specifically bound to the protein according to any one of claims 8 to 12 (claim 14), the antibody according to claim 14 which is a monoclonal antibody (claim 15), a host cell comprising an expression system expressing the protein according to any one of claims 8 to 12 (claim 16).

The present invention also relates to a non-human animal wherein a gene encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is excessively expressed (claim 17), a non-human animal wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome (claim 18), the non-human animal according to claim 18 having no reactivity against bacterial DNA having an unmethylated CpG sequence (claim 19), the non-human animal according to any one of claims 17 to 19 characterized in that a rodent animal is a mouse (claim 20).

The present invention also relates to a method of preparing a cell expressing a protein having reactivity against bacterial DNA having an unmethylated CpG sequence characterized in that the DNA according to any one of claims 1 to 7 is introduced into a cell wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome (claim 21), and a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG

sequence obtained by the method of preparing a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence according to claim 21 (claim 22).

The present invention also relates to screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: in vitro culturing a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the presence of a target substance, and measuring/evaluating TLR9 activity (claim 23), a screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: administering a target substance to a non-human animal wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome, and measuring/evaluating TLR9 activity of macrophages or spleen cells obtained from the non-human animal (claim 24), a screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: administering a target substance to a non-human animal wherein a gene encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is excessively expressed, and measuring/evaluating TLR9 activity of macrophages or spleen cells obtained from the non-human animal (claim 25), a screening method for an agonist or an antagonist of a protein having reactivity against bacterial DNA having the unmethylated CpG sequence according to either of claims 24 or 25 using a mouse as a non-human animal (claim 26).

The present invention also relates to an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence obtained by the screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence according to any one of claims 23 to 26 (claim 27), a pharmaceutical composition comprising whole or part of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence as an active component (claim 28), a pharmaceutical composition comprising the agonist or antagonist according to claim 27 as an active component (claim 29), a kit used to diagnose diseases related to the deletion, substitution and/or addition in a sequence of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated

CpG sequence comprising the DNA according to claim 3, which can compare a sequence of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in a test body with a sequence of bases in the DNA according to claim 3 (claim 30).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a gene maps of TLR9 knockout mice in the present invention and wild-type mice.

FIG. 2 shows the result of Southern blot analysis of TLR9 knockout mice in the present invention.

FIG. 3 shows the result of Northern blot analysis of spleen cells from TLR9 knockout mice in the present invention.

FIG. 4 shows the result of comparing the sequence of amino acids from TLR9 knockout mice in the present invention and the sequence of amino acids from wild-type mice.

FIG. 5 shows the result of measurement of TNF α , IL-6 or IL-12 production induced by CpG ODN, PGN or LPS in TLR9 knockout mice in the present invention and in wild-type mice.

FIG. 6 shows the result of cellular proliferation response induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

FIG. 7 shows the result of measurement of IL-12 production induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

FIG. 8 shows the result of expression of CD40, CD80, CD86, and MHC class II induced by CpG ODN or LPS in TLR9 knockout mice in the present invention and in wild-type mice.

FIG. 9 shows the result of activation of NF- κ B induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

FIG. 10 shows the result of activation of JNK induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

FIG. 11 shows the result of activation of IRAK induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

BEST MODE TO CARRY OUT THE PRESENT INVENTION

As bacterial DNA comprising an unmethylated CpG sequence in the present invention, any DNA derived from bacteria such as an oligodeoxynucleotide having an unmethylated CpG motif which activates immune cells such as T-cells, B-cells and antigen-presenting cells, and induces immune response can be used such as DNA derived from bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Serratia marcescens*, *Shigella flexneri*, *Vibrio cholerae*, *Salmonella Minnesota*, *Porphyromonas gingivalis*, *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Nocardia coeliaca*, *Streptococcus pneumoniae*.

As a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG motif, there are no particular restrictions as long as the protein can specifically recognize bacterial DNA with an unmethylated CpG sequence, and can be exemplified by human-derived TLR9 shown in Seq. ID No. 2 in the list of sequence, a protein which comprises a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in a sequence of amino acids shown in Seq. ID No: 2, and which specifically recognizes bacterial DNA having the unmethylated CpG sequence, or their recombinant proteins. The receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence can be prepared by well known methods based on the information of the DNA sequence and others.

DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of the present invention includes DNA encoding human-derived TLR9 shown in Seq. ID No: 2 in the list of sequence such as the one shown in Seq. ID No: 1, DNA comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in a sequence of amino acids shown in Seq. ID No: 2, and which can specifically recognize bacterial DNA having the unmethylated CpG sequence mentioned above, or DNA hybridized with the DNA under stringent conditions and encoding a protein that can specifically recognize bacterial DNA having the unmethylated CpG sequence mentioned above. These can be prepared by well known methods based on the information of DNA sequence such as mouse RAW264.7 cDNA library or 129/SvJ mouse gene library for mouse-derived TLR9.

Further, it is possible to obtain DNA encoding a receptor protein specifically recognizing bacterial DNA having an immune-inducing unmethylated CpG sequence which has the same effect as TLR9, a receptor protein, by hybridizing mouse-derived DNA library

with part or whole of a sequence of bases shown in Seq. ID No: 1 or its complementary sequence under stringent conditions to isolate the DNA hybridized with the probe. Conditions on hybridization to obtain the DNA can, for example, be hybridization at 42°C and wash treatment at 42°C with a buffer containing 1% \times SSC and 0.1% of SDS, and more preferably be hybridization at 65°C and wash treatment at 65°C with a buffer containing 0.1 \times SSC and 0.1% of SDS. Furthermore, beside the temperature conditions mentioned above, there are various factors effecting the stringency of hybridization, and it is possible for a person skilled in the art to realize the stringency equivalent to the stringency of hybridization illustrated above.

A fusion protein in the present invention can be the one obtained by combining a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence derived from mouse, human, and others with a marker protein and/or a peptide tag. A marker protein can be any marker protein previously well known, and can be exemplified by alkaline phosphatase, Fc region of an antibody, HRP, GFP and others. As a peptide tag in the present invention, it can be concretely exemplified by previously well-known peptide tags such as Myc tag, His tag, FLAG tag, GST tag. The fusion protein can be produced by a normal method, and is useful in purifying a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence by using affinity of Ni-NTA and His tag, detecting a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, measuring of the amount of antibodies against a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence and as a research reagent in other relevant fields.

As an antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention, it can be concretely exemplified by immune-specific antibodies such as a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a single-chain antibody, a humanized antibody. These antibodies can be produced by a normal method by using a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence mentioned above as an antigen, and a monoclonal antibody is preferable in its specificity among them. The antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence such as a monoclonal antibody and others is useful, for example, in diagnosing diseases caused by the mutation or deletion of TLR9 or

elucidating the molecular mechanism controlling TLR9.

An antibody against a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence can be produced by administering a fragment containing a receptor protein or an epitope specifically recognizing bacterial DNA having the unmethylated CpG sequence in animals (preferably, non-human), or a cell expressing the protein on the surface of its membrane by a conventional protocol, and any method can be used such as hybridoma method (Nature 256, 495-497, 1975), trioma method, human B cell hybridoma method (Immunology Today 4, 72, 1983), and EBV-hybridoma method (MONOCLONAL ANTIBODIES AND CANCER THERAPY, 77-96, Alan R. Liss, Inc., 1985), which are used for preparing monoclonal antibodies and brings an antibody produced by the cultured successive cell lines. The following explains a method of producing a monoclonal antibody specifically bound to mouse-driven TLR9, that is, an mTLR9 monoclonal antibody, with mouse-driven TLR9 as an example of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence.

The mTLR9 monoclonal antibody can be produced by a normal method of culturing hybridoma producing mTLR9 monoclonal antibody in vivo or in vitro. For example, in an in vivo systems they can be obtained by culturing in the visceral cavity of rodents, preferably of mice or rats, and in an in vitro system they can be obtained by culturing in a medium for culturing animal cells. A medium used for culturing hybridoma in an in vitro system can be exemplified by cell culture media such as RPMI1640 or MEN and others comprising antibiotics such as streptomycin or penicillin.

The hybridoma producing mTLR9 monoclonal antibody can be produced by immunizing BALB/c mouse with TLR9, a receptor protein obtained from mouse and others, fusing a spleen cell from an immunized mouse and a mouse NS-1 cell (ATCC TIB-18) by a normal method, and screening them by immunofluorescence staining patterns. A method of separating/isolating the monoclonal antibody can be any one as long as it is a method usually used for purifying proteins, and liquid chromatography such as affinity chromatography and others can be a concrete example.

It is also possible to apply the method of a single-chain antibody (US Patent No. 4946778) to produce single-chain antibodies against receptor proteins specifically recognizing bacterial DNA having the above-mentioned unmethylated CpG sequence of the present invention. Further, it is possible to use transgenic mice or other mammals and the like to

express humanized antibodies, isolate/identify the clones expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence by using the antibodies, and purify the polypeptides by affinity chromatography. The antibodies against receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence are useful in elucidating the molecular mechanism of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence.

It is also possible to carry out a functional analysis of a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence by using fusion proteins obtained by fusing proteins labeled with fluorescent substances such as FITC (fluorescein isothiocyanate) or tetramethylrhodamine isocyanate, fusion proteins labeled with radio isotopes such as ^{125}I , ^{32}P , ^{35}S or ^3H , enzymes such as Alkaline phosphatase, peroxidase, β -Galactosidase or Phycoerythrin, or fluorescent proteins such as Green Fluorescent Protein (GFP). A method of immunoassay can be exemplified by RIA, ELISA, fluorescence antibody method, plaque forming cell assay, spot method, hemagglutination reaction method, Ouchterlony Method, and others.

The present invention relates to a host cell comprising an expressing system that can express a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence. Introduction of a gene encoding a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence into a host cell can be carried out by the methods described in a number of standard laboratory manuals such as in Davis et al. (BASIC METHODS IN MOLECULAR BIOLOGY, 1986) and Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989), such as calcium phosphate transfection, DEAE-dextran-mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, infection and others. A host cell can be exemplified by bacterial prokaryotes such as Escherichia coli, Streptomyces, Bacillus subtilis, Streptococcus, Staphylococcus and others, fungal cells such as yeast and Aspergillus, insect cells such as Drosophila S2 or Spodoptera Sf9 and others, and animal and plant cells such as L cell, CHO cell, COS cell, Hela cell, C127 cell, BALB/c3T3 cell (including mutant strains lacking dihydrofolate reductase, thymidine kinase or others), BHK 21 cell, HEK293 cell, Bowes Melanoma cell, oocytes, and others.

Further, the expression system can be any one as long as it is a system that can express

a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence in a host cell, and can be exemplified by expression systems derived from chromosome, episome and virus, such as vectors derived from bacterial plasmid, yeast plasmid, papovavirus such as SV40, vaccinia virus, adeno virus, fowl poxvirus, pseudorabies virus, or vectors derived from retrovirus, vectors derived from bacteriophage or transposon or their combinations, which can be exemplified by plasmids such as cosmid and phagemid, which are derived from genetic factors of plasmids and bacteriophage. These expressing systems may comprise a control sequence that not only causes expression but also regulates expression.

A receptor protein specifically recognizing a host cell comprising the expressing system or a cell membrane of the cell, bacterial DNA comprising an unmethylated CpG sequence obtained by culturing, and the cell can be used for the screening methods of the present invention as mentioned below. For example, a method described in F. Pietri-Rouxel et al. (Eur. J. Biochem., 247, 1174-1179, 1997) can be used as a method for obtaining cell membrane, and well known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion- or cation-exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography, preferably high-performance liquid chromatography can be used to collect a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence from the cell culture and to purify it. Specifically, it is possible to obtain a receptor protein specifically recognizing the bacterial DNA having an unmethylated CpG sequence by using a column to which a receptor protein antibody specifically recognizing bacterial DNA having the anti-unmethylated CpG sequence of anti-TLR9 monoclonal antibodies and others is bound, or in case an ordinary peptide tag is bound to a receptor protein such as TLR9 etc. specifically recognizing a column to which a substance having an affinity with a peptide tag is bound for affinity chromatography.

A non-human animal excessively expressing a gene encoding a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence mentioned above in the present invention can be a non-human animal producing a large amount of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence compared with wild-type non-human animals. Further, a non-human animal whose gene function encoding a receptor protein specifically recognizing bacterial DNA

having an unmethylated CpG sequence is deleted on the chromosome is a non-human animal wherein part or whole of genes encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence on the a chromosome are inactivated by genetic mutations such as damaged, deleted, substituted, and others, and which lost a function of expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence. Although the non-human animal used in the present invention can be exemplified by a non-human animal including rodents such as rabbits, mice, rats and others, it is not restricted to the animals.

Further, refractory against bacterial DNA having an unmethylated CpG sequence in the present invention means that the reactivity against stimuli by bacterial DNA shown by an organism, or a cell, a tissue or an organ constituting the organism is declined or almost totally lost. Therefore, a non-human animal with refractory against bacterial DNA having an unmethylated CpG sequence in the present invention is a non-human animal such as mice, rats, or rabbits, wherein the an organism's reactivity against bacterial DNA, or a cell, a tissue or an organ constituting the organism is declined or almost totally lost. Further, stimuli by bacterial DNA can be exemplified by an in vivo stimulus caused by administering bacterial DNA to an organism, or an in vitro stimulus caused by contacting cells separated from an organism with bacterial DNA. Concretely, a non-human animal such as TLR9 knockout mice wherein TLR9 gene functions are destroyed on the chromosome can be an example.

A homozygote non-human animals born following Mendel's Law includes mice deficient of or excessively expressing receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence and their wild-type littermates, and it is preferable to use wild-type non-human animals, that is, the same kind of animal as a non-human animal wherein gene functions encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence are destroyed or are excessive, more preferably their littermate animals, for example, during the screening of the present invention described below because accurate comparative experiments can be carried out at the level of individuals by using the homozygote non-human animals with its receptor proteins destroyed or the one with receptor proteins expressing excessively or the wild-type non-human animals born from the same mother at the same time. In the following, a method of producing non-human animals wherein gene functions encoding a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence are destroyed or excessively

expressed on the chromosome is explained using knockout mice or transgenic mice whose receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence as an example.

For example, as for a mouse wherein gene functions encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence are destroyed on a chromosome such as TLR9, that is, a knockout mouse lacking receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence, gene fragments obtained from mouse gene library by a method of PCR or the like are used to screen genes encoding receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence, subclone a gene encoding a receptor protein specifically recognizing bacterial DNA having the screened unmethylated CpG sequence with viral vectors and others, and specified by DNA sequencing. Whole or part of the gene in the clone encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is substituted with pMC1 neo gene cassette and others, and a targeting vector is produced by introducing diphtheria toxin A fragments (DT-A) genes or herpes simplex virus thymidine kinase (HSV-tk) genes and others on 3'-end side.

The produced targeting vector is linearized, introduced into ES cells by electroporation method and others, homologous recombination is performed, and ES cells which has caused homologous recombination by antibiotics such as G418 or gancyclovir (GANC) and others are selected from the homologous recombinants. It is preferable to confirm by Southern blot technique that the selected ES cells are targeted recombinants. The clones of the confirmed ES cells are introduced to mouse blastocysts by microinjection, and the blastocysts are returned to recipient mice, and chimera mice were produced. The chimera mouse was intercrossed with a wild-type mouse to produce a heterozygote mouse, and the heterozygote mice are intercrossed to produce a knockout mouse lacking a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention. Further, a method of confirming whether knockout mice lacking a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is obtained, for example, may be examined by Northern blot technique, which isolates RNA from the mouse obtained by the method mentioned above, or the expression in the mice may be examined by Western blot technique.

The fact that the produced TLR9 knockout mouse is refractory against bacterial DNA

having an unmethylated CpG sequence can be confirmed by measuring the levels of the production of TNF- α , IL-6, IL-12, IFN- γ and others in the cells whose CpG ODN was contacted in vivo or in vitro with immune cells such as macrophages, mononuclear cells, dendritic cells from TLR9 knockout mice, the proliferation of response of spleen B cells, the expression of antibodies such as CD40, CD80, CD86, MHC class II on the surface of spleen B cells, and the activation of molecules on the signal transduction pathway of NF- κ B, JNK, IRAK and others. The knockout mice lacking TLR9 in the present invention can be used to elucidate functional mechanisms of bacterial DNA and others having an unmethylated CpG sequence and to developing vaccine against bacterial infections.

Transgenic mice lacking receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence can be generated by constructing introduced genes by fusing chicken β actin, mouse neurofilament, promoters such as SV40, and rabbit β -globin, polyA such as SV40 or intron with cDNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence such as TLR9, microinjecting the introduced genes to pronucleus of mouse fertilized eggs, transplanting the obtained cells to an oviduct of recipient mice after culturing them, then breeding the transplanted animals, and selecting child mice having the cDNA from born child mice. Further, selection of the child mice having cDNA can be performed by dot hybridization wherein crude cDNA was extracted from mouse tails and others, and genes encoding receptor proteins specifically recognizing bacterial DNA having an introduced unmethylated CpG sequence is used as a probe, or PCR method using specific primers and others.

Further, the use of whole or part of DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention enables us to prepare cells effective for genetic treatments for diseases caused by the deletion or abnormality of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence. Methods of preparing the cells in the present invention can be exemplified by a method wherein part or whole of the DNA in the present invention is introduced into cells lacking gene functions encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence on the chromosome by transfection and others, and thus obtaining a cell expressing receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence. It is preferable to use a cell in which the DNA and others is integrated onto the chromosome and shows TLR9

activity in a stable manner, particularly as a cell expressing receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence.

Furthermore, the use of DNA encoding receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence, antibodies against receptor proteins specifically recognizing bacterial DNA having a fused unmethylated CpG sequence comprising a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence bound to a marker protein and/or a peptide tag, a host cell comprising an expression system which can express a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, non-human animals excessively expressing genes encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence, non-human animals lacking gene functions encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence on a chromosome, cells expressing receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence enables us to screen agonists or antagonists of the receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention, or suppressing or promoting substances reactive to bacterial DNA having an unmethylated CpG sequence. What is obtained by the screening may be suppressing or promoting substances against bacterial infected diseases, suppressing agents, preventing agents or remedies against allergic diseases or cancers, agents suppressing or promoting side effects in genetic therapy or the like, or substances useful for diagnosing/treating diseases or the like caused by the deletion or abnormality of TLR9 activity.

Although the TLR activities can concretely be exemplified by a function of reacting specifically to bacterial DNA having an unmethylated CpG sequence and transmitting signals into cells, and a signal transduction function is a function of producing cytokines such as TNF- α , IL-6, IL-12, IFN- γ or the like, a function of producing nitrous acid ion, a function of proliferating cells, a function of expressing antibodies such as CD40, CD80, CD86, MHC class II and others on the surface of cells, and a function of activating molecules in signal transduction pathway of TLR9 such as NF- κ B, JNK, IRAK and others, it is not limited to these functions.

A screening method of agonists or antagonists of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention

human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence are destroyed on a chromosome first, then administrating the target substances to the non-human animals, and measuring/evaluating the levels of macrophage activities or spleen cell activities in the non-human animals. Although as bacterial DNA having an unmethylated CpG sequence used in the screening methods, it is preferable to use CpG ODN (TCC-ATG-ACG-TTC-CTG-ATG-CT: Seq. ID No: 5), it is not limited to this.

The present invention also relates to a kit used to diagnose diseases relating to the activity or expression of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence by comparing a sequence of DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in a test body with a sequence of DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention. The detection of mutated DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence can be carried out by detecting genetically mutated individuals at the level of DNA, and is effective for diagnosing diseases caused by hypotypic expression, hypertypic expression or mutated expression of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence. Although a test body used in the detection can concretely be exemplified by genomic DNA of cells from subjects obtainable by biopsy from blood, urine, saliva, tissue and others, RNA, or cDNA, it is not limited to these. In using the test body, it is possible to use the ones amplified by PCR and others. The deficiency or insertional mutation in sequences of bases can be detected by the changes of amplified products in size compared with normal genes, and point mutation can be identified by hybridizing the amplified DNA with the gene encoding receptor proteins specifically recognizing bacterial DNA having labeled unmethylated CpG sequence. It is possible to diagnose or conclude diseases relevant to activity or expression of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence by detecting mutation of a gene encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence.

The present invention also relates to a probe diagnosing a disease related to activities or expressions of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising whole or part of antisense chain of DNA or RNA

encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, and a kit used to diagnose diseases relating to activities or expressions of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising an antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of the probe and/or in the present invention. A probe used for the diagnosis is whole or part of an antisense chain of DNA (cDNA) or RNA (cRNA) encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, and there is no limitations on the probe as long as it is long enough (at least 20 bases or more) to establish as a probe. In order to make an antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of the probe and/or in the present invention an active component of a medicine diagnosing diseases such as bacterial infection and others, it is preferable to dissolve it into appropriate buffers or sterilized water in which a probe is not decomposed. Further, it is possible to use the clinical test pharmaceuticals to diagnose a patient's symptoms such as bacterial infection diseases and others in the ways such as immunofluorescence (Dev. Biol. 170, 207-222, 1995, J. Neurobiol. 29, 1-17, 1996), In situ hybridization (J. Neurobiol. 29, 1-17, 1996), or in situ PCR or others.

A pharmaceutical composition of the present invention can be any one as long as it comprises whole or part of the receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of TLR9 or others, or comprises an agonist or an antagonist of the receptor protein. Concretely, vaccines against bacterial infectious diseases, vaccines against cancers, treating medicine for patients having allergies such as bronchial asthma, reversal agents, suppressing agents, inhibiting agents and others for side effects by the existence of a CpG motif inhibiting genetic treatments or treatments using antisenseoligonucleotides can be exemplified.

As mentioned above, a kit testing diagnoses relevant to the deletion, substitution and/or addition of DNA sequence encoding a receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence of the present invention can be any one as long as it comprises DNA encoding TLR9, and comparing a sequence of bases of DNA encoding the TLR9 with a sequence of bases of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in a tested body enables us to diagnose diseases related to deletion, substitution and/or addition of DNA sequence

encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence such as cancer, allergy, infectious diseases and others.

In the following, the present invention will further be explained with concrete examples. However, the technical scope of the present invention is not limited in any way by the following examples.

Example 1: Cloning of TLR9

As a result of a GenBank search using the information of DNA sequence of human TLR4, a mouse EST having a significant homology (Registration No. AA273731; mouse) was found. Using PCR amplified mouse EST as a probe, mouse RAW 264.7 cDNA library was screened and a full length cDNA clone shown in Seq. ID No. 3 comprising the complete TLR9 open reading frame was isolated. Performing a GenBank search based on the information of DNA sequence of the mouse TLR9, a human genomic sequence having a high level of homology was found. Based on the human genomic sequence, cDNA ends were amplified to isolate cDNA of the full length human TLR9 having a sequence of bases in Seq. ID No. 1 from U937 cells (J. Immunol. 163, 5039-5048, 1999).

Example 2: Production of TLR knockout mice

The TLR9 genomic DNA was isolated from 129/SvJ mouse genomic library (Stratagene), subcloned in pBluescript II SK(+) vectors (Stratagene), and characterized by restriction enzyme mapping and DNA sequencing analysis. The targeting vector was constructed by replacing a 1.0 kb fragment encoding part of LRR (leucine-rich repeat) region with a neomycin-resistance gene cassette (pMC1-neo; Stratagene), and a herpes simplex virus thymidine kinase (HSV-TK) was inserted for negative selection (Fig. 1). The targeting vector was linearized, and was electroporated into embryonic stem cells (ES cells) of E14-1, then 292 pieces of clones showing G418 and gancyclovir resistance were selected, and 14 pieces of clones were screened by PCR and Southern blotting.

Chimeric mice were produced by microinjecting 3 pieces of targeted ES clones comprising mutated TLR9 allele into C57BL/6 mouse blastocysts. The male chimeric mice were intercrossed with C57BL/6 female mice to produce a heterozygote F1 mouse, and a homozygote mice (TLR9 knockout mouse: TLR9^{-/-}) was obtained by intercrossing heterozygote F1 mouse (Fig. 2). To confirm that the obtained mouse was homozygote,

various genomic DNA extracted from a mouse tail was digested by ScaI to perform Southern blotting using the probe shown in Fig. 1. The TLR9 knockout mice (TLR9^{-/-}) of the present invention were produced following Mendel's law, and had not shown remarkable abnormality for 12 weeks.

To confirm that the inactivation of TLR9 arises by mutation, total RNA (10 μ g) extracted from spleen cells from wild-type mice (+/+) and TLR9 knockout mice (-/-) was electrophoresed, and transferred to nylon membranes, Northern blotting was performed with the use of cDNA specific to TLR9 c-terminal fragments or N-terminal fragments labeled with [³²P], or β -actin (Fig. 3). The result shows that N-terminal fragments of TLR9 mRNA were not detected from the spleen cells of TLR9 knockout mice. Further, with a C-terminal fragment as a probe, almost the same size of Tlr9 transcripts derived from mutated mice as the ones from wild-type mice were detected. However, the amount of the production was small. Then, RT-PCR was performed using mRNA of spleen cells obtained from mutated mice to sequence the obtained products. The result shows that the Tlr9 gene transcript comprises neo gene, and stop codons appear in a N-terminal domain of TLR9 by inserting the neo, and functional TLR9 proteins does not appear in mutated mice (Fig. 4). Further, as a result of examining lymph cells from TLR9 by flowcytometry knockout mice, no abnormal compositions were found.

Example 3: Preparation of peritoneal macrophages

2ml of 4% thioglycolic acid medium (DIFCO) was injected to each peritoneum of wild-type mice and TLR9 knockout mice (TLR9^{-/-}), peritoneal exudation cells were isolated from peritonea from each mouse after 3 days, the cells were cultured in RPMI1640 medium to which 10% of fetal bovine serum (GIBCO) was added at 37°C for 2 hours, and remove the unattached cells by washing with ice-chilled Hank's buffered salt solution (HBSS; GIBCO), and the attached cells were used as peritoneal macrophages in the following experiments.

Experiment 4: Response to bacterial DNA having an unmethylated CpG sequence in TLR9 knockout mice

It has recently been shown that the response of CpG ODN (oligodeoxynucleotide) is dependent on MyD88, an adopter protein in a signaling transduction pathway mediating TLR. Although the MyD88 knockout mice do not show response to CpG ODN, TLR2 knockout

mice or TLR4 knockout mice show normal response to it. This shows that CpG ODN recognizes TLRs other than TLR2 and TLR4, and then the response of a TLR9 knockout mouse against CpG ODN was examined. First, the amount of producing inflammatory cytokines in peritoneal macrophages were measured in the following way.

The macrophages prepared in Example 3 are co-cultured with various concentrations of CpG ODN shown in Fig. 5 (0.1 or 1.0 μ M; TIB MOLBIOL; TCC-ATG-ACG-TTC-CTG-ATG-CT), PGN (10 μ g/ml; Sigma and Fluka; derived from *Staphylococcus aureus*), LPS (1.0 μ g/ml; Sigma; derived from *Salmonella minnesota* Re-595) in the presence or absence of IFN γ (30 unit/ml). The concentrations of TNF α , IL-6 and IL-12 p40 in the supernatants after culturing were measured by ELISA, and the results are shown in Fig. 5. The results show that the macrophages from wild-type mice (Wild-type) produce TNF α , IL-6 and IL-12 in response to CpG ODN, and further stimulation by IFN γ and CpG ODN increases the amount of producing TNF α , IL-6 and IL-12. However, the macrophages derived from TLR9 knockout mice (TLR9^{-/-}) did not produce a detectable level of inflammatory cytokines in response to CpG ODN even in the presence of IFN γ . Further, it was found that the macrophages derived from wild-type mice and TLR9 knockout mice produce almost the same level of TNF α , IL-6 and IL-12 in response to LPS or PGN (Fig. 5). Each experimental result shows the average level of n=3. N.D. in the figures means not detected.

Response of spleen cells from wild-type mice (Wild-type) and TLR9 knockout mice (TLR9^{-/-}) against CpG ODN or LPS was also examined. The spleen cells from each mouse (1×10^5) were isolated to culture in 96 well plates by CpG DNA or LPS of various concentrations shown in Fig. 6, and the spleen cells were stimulated. 40 hours later from culturing, 1 μ Ci of [³H]-thymidine (Dupont) was added, and then further cultured for 8 hours. The amount of uptaking [³H]-thymidine was measured by β scintillation counter (Packard) (Fig. 6). The results that although the spleen cells from wild-type mice promote cell proliferating reactions depending on the amount of administrating CpG ODN or LPS, the spleen cells from TLR9 knockout mice did not show any cell proliferating reaction by CpG ODN even with the stimulus of any concentration of CpG ODN. Further, the amount of expressing Major Histocompatibility Complex (MHC) class II on the surface of B cells derived from wild-type mice in response to CpG ODN was increased. However, such increase of the amount of expressing MHC class II induced by CpG ODN in B cells derived

from TLR9 knockout mice was not observed. These facts show that the macrophages or B cells from TLR9 knockout mice specifically lack the response against CpG ODN.

Next, it is well known that DNA derived from bacteria comprising CpG ODN potentially stimulates dendritic cells, and supports the development of Th1 cell (EMBO J. 18, 6973-6982, 1999, J. Immunol. 161, 3042-3049, 1998, Proc. Natl. Acad. Sci. USA 96, 9305-9310, 1999). Then, the production of CpG ODN-inducing cytokines and the upregulation of the surface molecule of dendritic cells derived from bone marrow were examined. The bone marrow cells from wild-type mice (Wild-type) or TLR9 knockout mice (TLR9^{-/-}) were cultured with 10ng/ml mouse granulocyte macrophage-colony stimulating factor (Peprotech) in RPMI1640 medium supplemented with 10% fetal bovine serum (J. Exp. Med. 176, 1693-1702, 1992), at day 6 of the culture, immature dendritic cells were harvested and cultured in the presence or absence of 0.1 μ M CpG ODN or 0.1 μ g/ml LPS in RPMI1640 medium supplemented with 10% fetal bovine serum for 2 days. After the culture, the concentration of IL-12 p40 in the supernatants was measured by ELISA (Fig. 7). The result shows that the dendritic cells derived from wild-type mice produced IL-12 in response to CpG ODN while the dendritic cells derived from TLR9 knockout mice did not induce the production of IL-12 in response to CpG ODN.

After culturing in RPMI supplemented with 10% fetal bovine serum was cultured which contains 10ng/ml mouse granulocyte macrophage-colony stimulating factor (Peprotech), the dendritic cells harvested at day 6 were stained with biotinylated antibodies against CD40, CD80, CD86 or MHC class II, developed with streptavidine labeled with phycoerythrin (PE; PharMingen). The cells were examined by using a FACSCalibur with CELLQuest software (Becton Dickinson) (Fig. 8). The result shows that stimulation by CpG ODN promotes the expression of CD40, CD80, CD86 and MHC class II on the surface of dendritic cells derived from wild-type mouse while it does not promote the expression of these molecules on the surface of dendritic cells derived from TLR9 knockout mouse by the stimulation of CpG ODN (Fig. 8). The dendritic cells from wild-type mice and from TLR9 knockout mouse show similar responses in response to LPS. This result shows that TLR9 is a receptor essential for cell response to CpG ODN.

Example 5: activation of NF- κ B, JNK and IRAK in response to
CpG ODN of macrophages derived from TLR9 knockout mice

It is known that signaling via TLRs activates IRAK, a serine-threonine kinase mediated by MyD88, an adaptor molecule, and subsequently activates MAP kinase and NF- κ B (Immunity 11, 115-122, 1999). Whether CpG ODN activates the intracellular signaling or not was examined. The peritoneal macrophages (1×10^6 cells) from wild-type and TLR9^{-/-} mice in Example 3 were stimulated by $1.0 \mu\text{M}$ of CpG ODN or $1.0 \mu\text{g/ml}$ of LPS from *Salmonella minnesota* Re-595 for the periods indicated in Fig. 9, nucleoproteins were extracted from the macrophages obtained from each mouse to be incubated together with a specific probe comprising NF- κ B DNA-binding sites, electrophoresed, and then visualized by autoradiography (Fig. 9).

The result shows that when stimulated by CpG ODN, the macrophages derived from wild-type mice increased NF- κ B DNA-binding activity while the macrophages derived from TLR9 knockout mice did not increase NF- κ B DNA-binding activity. When stimulated by LPS, the macrophages derived from TLR9 knockout mice and the macrophages derived from the wild-type mice show similar NF- κ B activities.

The result shows that the macrophages derived from a TLR9 knockout mouse specifically lack NF- κ B activity by the induction of CpG ODN. The arrows in the figures indicate the sites of the compounds of NF- κ B and specific probes, and the arrowheads indicate the sites of specific probes only.

As shown above, the macrophages from wild-type mice and TLR9 knockout mice stimulated by CpG ODN or LPS for the periods indicated in Fig. 10 and Fig. 11 were dissolved into a solvent buffer (a buffer comprising 1.0% Triton X-100, 137mM of NaCl, 20mM of Tris-HCl, 5mM of EDTA, 10% glycerol, 1mM of PMSF, $20 \mu\text{g/ml}$ of aprotinin, $20 \mu\text{g/ml}$ of leupeptin, 1mM of Na_3VO_4 and 10mM of β -glycerophosphate at the final concentrations; pH8.0), the cell lysates were immunoprecipitated with anti-JNK antibody (Santa Cruz) or anti-IRAK antibody (Hayashibara Seikagaku Kenkyujo Kabushiki Kaisha). As described in a reference (Immunity 11, 115-122, 1999), the JNK activity and IRAK activity were measured by in vitro kinase assay using GST-c-Jun fusion protein (GST-c-Jun) as a substrate (top figures of Fig 10 and Fig.11; GST-c-Jun, Auto).

The cell lysates were separated by SDS-polyacrylamide gel electrophoresis to transfer them onto a nitrocellulose membrane and blotted the membrane with anti-JNK antibody (Santa Cruz) or anti-IRAK antibody (Transduction Laboratories) to visualize using an

enhanced chemiluminescent system (Dupont) (bottom figures of Fig. 10 and Fig. 11; WB). The result shows that CpG ODN activates JUN and IRAK of the macrophages derived from wild-type mice while it does not activate JUN and IRAK of the macrophages derived from TLR9 knockout mice (Fig. 10 and Fig.11). It is therefore found that the signaling transduction mediated by CpG ODN depends on TLR9.

INDUSTRIAL APPLICABILITY

Bacteria-derived DNA comprising an unmethylated CpG motif significantly activates immune cells and induce Th1 response, while a receptor recognizing such bacterial DNA remained unknown. The present invention has revealed a receptor of oligonucleotides comprising an unmethylated CpG sequence of bacterial DNA and will enable us to elucidate a receptor protein TLR9, a member of TLR family, specifically recognizing bacterial DNA having an unmethylated CpG sequence, the genetic DNA encoding it or others, which will be useful to diagnose and treat bacterial diseases and others. The use of the TLR9 knockout animals will also enable us to elucidate functional mechanisms of DNA derived from bacteria at the molecular level.